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<p>(21) International Application Number: PCT/US96/02664</p> <p>(22) International Filing Date: 26 February 1996 (26.02.96)</p> <p>(30) Priority Data: 08/412,231 10 March 1995 (10.03.95) US</p> <p>(71) Applicant (for all designated States except US): BOARD OF REGENTS UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES [US/US]; 4301 Jones Bridge Road, Bethesda, MD 20814-4799 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): O'BRIAN, Alison [US/US]; 5514 Charicote Road, Bethesda, MD 20817 (US). LINDGREN, Susanne, Ward [US/US]; Dept. of Microbiology & Immunology, OHSU, L220, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201 (US). PERERA, Liyanage, P. [US/US]; 306 Farragut Avenue, Rockville, MD 20851 (US). STROCKBINE, Nancy, A. [US/US]; 3035 Stone Mountain Street, Lithonia, GA 30058 (US). MELTON-CELSA, Angela, Ruth [US/US]; 20516 Brandywine Court, Sterling, VA 20165 (US).</p>	<p>(74) Agent: BELLAMY, Werten, F., W.; Intellectual Property Law Division, Office of the Judge Advocate General, DA, Suite 700, 901 North Stuart Street, Arlington, VA 22203-1837 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>	
<p>(54) Title: DETECTION OF SHIGA-LIKE TOXINS OF ENTEROHEMORRHAGIC ESCHERICHIA COLI</p> <p>(57) Abstract</p> <p>A rapid, sensitive, non-radioactive diagnostic kit for the direct detection of both Shiga-like toxin, type I, and Shiga-like toxin, type II, produced by enterohemorrhagic <i>Escherichia Coli</i> in food and clinical samples. This diagnostic kit is comprised of a monoclonal antibody capable of detecting Shiga-like toxin, type I, and a monoclonal antibody capable of detecting Shiga-like toxin, type II, together with a chemiluminescent detection reagent with a sensitivity enhancer.</p>		

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1 DETECTION OF SHIGA-LIKE TOXINS
2 OF ENTEROHEMORAGIC ESCHERICHIA COLI

3 STATEMENT OF GOVERNMENT INTEREST

4 The invention described herein may be
5 manufactured, used and licensed by or for the
6 Government for governmental purposes without payment to
7 us of any royalties thereon.

8 BACKGROUND OF THE INVENTION

9 This invention generally relates to the detection
10 of Shiga-like toxins by means of a fast, highly
11 sensitive assay without necessity of tissue culture.
12 As such, the invention presents a novel means of
13 testing for the presence of toxins in food products and
14 clinical stool samples, and may be used to quantify the
15 amount of toxin occurring in the sample.

16 Infection with Enterohemorrhagic *Escherichia coli*
17 (EHEC) is associated with food-borne outbreaks of
18 diarrhea, hemorrhagic colitis and the hemolytic uremic
19 syndrome. Hemorrhagic colitis is characterized by
20 severe abdominal pain with watery diarrhea. This is
21 followed by grossly bloody diarrhea without fever
22 (Riley, L.W., 1987, The epidemiologic, clinical, and
23 microbiological features of hemorrhagic colitis, Ann.
24 Rev. of Microbiology 41:383-407). The symptoms

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1 typically last from four to eight days. The illness is
2 usually self-limiting. Hemolytic uremic syndrome
3 associated with EHEC is characterized by a
4 thrombocytopenia, microangiopathic hemolytic anemia and
5 acute renal failure (Levin, et al., 1989, Hemolytic
6 uremic syndrome, Adv. Pediatric Infectious Disease
7 4:51-82). The illness occurs predominantly in children
8 under four years of age.

9 EHEC infections are predominantly associated with
10 industrialized and developing countries. Reports of
11 EHEC infections have suggested transmission through a
12 variety of food products of animal origin, including
13 meat, poultry, and animal products such as
14 unpasteurized milk. The transmission of EHEC may also
15 be accomplished by person to person contact and has
16 been reported as the source of outbreaks at day care
17 centers (Spika, et al., 1986, Hemolytic-uremic syndrome
18 associated with *Escherichia coli* O157:H7 in a day care
19 center, J. Pediatr. 109:287-291; Belongia, et al.,
20 1993, Transmission of *Escherichia coli* O157:H7
21 infection in Minnesota child day-care facilities, JAMA
22 269:883-888).

23 EHEC infections are particularly associated with
24 the developed and developing countries where
25 significant amounts of beef are consumed. In
26 particular, EHEC is associated with consumption of

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1 undercooked hamburger meat. One such instance involved
2 an outbreak in February 1993 in the western United
3 States apparently originating with consumption of
4 hamburger at a chain of fast food restaurants (Centers
5 for Disease Control, 1993, "Update: Multistate
6 outbreak of *Escherichia Coli* 0157:H7 infections from
7 hamburgers -- Western United States," MMWR Vol. 42, pp.
8 258-263).

9 A common characteristic of EHEC strains is the
10 production of Shiga-like toxins (SLTs), also known as
11 Vero toxins. Shiga-like toxins are multiple subunit
12 toxins, consisting of one enzymatically active A
13 subunit and five receptor-binding B subunits. Shiga-
14 like toxins have been categorized in two groups based
15 on binding property and immunological activity.

16 The first group, designated Shiga-like toxin type
17 I (SLT-I), includes the prototype toxin SLT-I and Shiga
18 toxin from *Shigella dysenteriae* type I. Shiga toxin
19 and SLT-I differ by only one amino acid and are
20 considered to be the same toxin. No other homologous
21 toxins are currently known to exist. Both SLT-I and
22 Shiga toxin use the glycolipid globotriaosylceramide
23 (Gb₃, Gal α 1-4Gal β 1-4Glc β 1-1Cer) as the functional
24 eucaryotic cell-surface receptor. Hereinafter, the
25 designation Shiga-like toxin type I or SLT-I will be
26 understood to include Shiga toxin produced by *Shigella*

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1 disenteriae type I. SLT-I can be neutralized by
2 antiserum to purified Shiga toxin and by monoclonal
3 antibodies to the B subunit to of SLT-I.

4 The second group, Shiga-like toxin type II, cannot
5 be neutralized by anti-SLT-I monoclonal or polyclonal
6 antisera. This group exhibits sequence and antigenic
7 variation. The prototype SLT-II toxin is produced by
8 EHEC 0157:H7. The prototype toxin shares 55 and 57
9 percent deduced amino acid sequence homology with SLT-I
10 A and B subunits respectively. Another member of the
11 group, SLT-IIv, is responsible for edema disease in
12 swine. SLT-IIv demonstrates 93 and 84 percent deduced
13 amino acid sequence homology with the prototype SLT-II
14 A and B subunits respectively. SLT-IIv more avidly
15 binds globotetraosylceramide (Gb₃, GalNAc β 1-3Gal α 1-
16 4Gal β 1-4Glc β 1-1Cer), whereas the other SLT-II toxins,
17 like their SLT-I counterparts, use Gb₃ as the cell
18 surface receptor.

19 Since 1990, additional Shiga-like toxins have been
20 described which are considered members of the SLT-II
21 group. This classification is based on their sequence
22 homology and immunological cross-reactivity with SLT-
23 II. The additional members include SLT-IIvha, SLT-
24 IIvhb and SLT-IIc. Those members which have been amino
25 acid sequenced are nearly 97 percent homologous to the
26 prototype SLT-II strain in both the A and B subunits.

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1 The potential for extensive outbreaks of
2 enterohemorrhagic colitis resulting from contaminated
3 food has produced the need for a fast, simple and
4 sensitive test for detection of all SLTs. To be
5 effective, any such test must be capable of detecting
6 the presence of all Shiga-like toxins. Existing tests
7 capable of detecting SLTs have failed to provide the
8 necessary rapidity, the requisite specificity or both.

9 One assay is specific for detecting SLT-II
10 produced by *E. Coli* 0157:H7 (Doyle, et al., 1987,
11 "Isolation of *Escherichia Coli* 0157:H7 from Retail
12 Fresh Meats and Poultry," Applied and Environmental
13 Microbiology, 53:2394-2396). This method requires
14 incubating a sample in an enrichment medium overnight.
15 The sample is then filtered through hydrophobic grid
16 membrane paper. The filter paper is then placed on a
17 nitrocellulose paper and the nitrocellulose paper is
18 again incubated overnight with an enrichment medium.
19 The toxins are then detected using an antibody to the
20 toxin and standard immunoblot procedures. The
21 procedure is time consuming and complex. Furthermore,
22 the procedure does not detect all SLTs.

23 Other assays are available for detection of EHEC,
24 particularly *E. Coli* 0157:H7. These assays detect the
25 presence of the organism rather than the toxin it

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1 produces. One such assay is described in United States
2 Patent No. 5,168,063. The antibody described in that
3 patent reacts with a protein in the outer membrane of
4 the EHEC having molecular weight of approximately
5 13,000 daltons. The patent also describes a method of
6 using the antibody in an assay for detecting the
7 presence of the EHEC. The assay, however, requires the
8 incubation of the sample believed to contain EHEC,
9 which is time-consuming and requires that the user have
10 adequate tissue culture facilities.

11 Further, previously known assays for EHEC and
12 their toxins have generally relied on radiolabeling as
13 a detection means because radiolabeling offered the
14 requisite degree of sensitivity. Radiolabeling has
15 several disadvantages, however. Radiolabeling involves
16 the use of hazardous agents, requiring the protection
17 of the user and the safe disposal of the waste.
18 Radiolabeling is also time consuming to conduct.

19 SUMMARY OF THE INVENTION

20 It is an object of the present invention to
21 provide a means for detecting both Shiga-like toxins,
22 type I (SLT-I) and type II (SLT-II) in a single assay.
23 It is also an object of the invention to provide a
24 means for detecting the presence of substantially all
25 enterohemorrhagic *E. Coli* which produce SLTs by means
26 of a single assay directed against the product toxins.

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1 It is further an object of this invention to
2 provide a means for rapidly detecting the presence of
3 SLT-producing EHEC in clinical samples, such as stool
4 samples. It is also an object of the invention to
5 provide a means for rapidly detecting the presence of
6 SLTs and SLT-producing EHEC in food samples.

7 It is further an object of this invention to
8 provide a means for detecting SLTs in food and clinical
9 samples which is highly sensitive and relatively safe
10 for the user. It is also an object of this invention
11 to provide a means for detecting SLTs which does not
12 involve tissue culture.

13 It is further an object of this invention to
14 provide diagnostic kits for assaying the presence of
15 both SLT-I and SLT-II in food or other samples. It is
16 an object of this invention to provide diagnostic kits
17 capable quantifying the level of toxin found in a
18 clinical or food sample.

19 This invention is a diagnostic kit for detecting
20 the presence of SLT-I and SLT-II, specifically in food
21 and clinical samples. The kit is comprised of two
22 heterologous monoclonal antibodies in an aqueous
23 solution and a sensitive chemiluminescent detection
24 reagent. One of the antibodies is produced from a
25 hybridoma formed by the fusion of a mouse myeloma line
26 and spleen cells from mice immunized with SLT-I and

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1 selected for its specificity for the SLT-I B subunit.
2 The other antibody is produced from a hybridoma formed
3 by the fusion of a mouse myeloma line and spleen cells
4 immunized with a toxoid derived from SLT-II and
5 selected for its specificity for the SLT-II A subunit.
6 The chemiluminescent detection reagent is a solution of
7 a chemiluminescing compound, an oxidant and a
8 sensitivity enhancer. In the presence of a peroxidase
9 enzyme which is conjugated either to a secondary
10 antibody or directly to the previously described
11 monoclonal antibodies, the chemiluminescing compound is
12 oxidized to an excited state, which emits a measurable
13 amount of light when returning to a non-excited state.
14 In order to produce the requisite sensitivity for the
15 detection of low to moderate amounts of SLT, a
16 sensitivity enhancer is included in the detection
17 reagent.

18 DETAILED DESCRIPTION OF THE INVENTION

19 The methods for preparing monoclonal antibodies
20 generally are well known to those in the art. In
21 preparation of monoclonal antibodies against SLT-I,
22 BALB/c mice are immunized with biologically active
23 Shiga toxin from *Shigella dysenteriae*, type 1, or SLT
24 from an SLT-I producing *E. Coli*, such as strain H30,
25 using methods well-known in the art. The mice are
26 sacrificed and their splenocytes are harvested. The

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1 splenocytes are fused to an appropriate mouse myeloma
2 cell line according to methods well-known in the art.
3 The hybridomas are then cultured and the cell culture
4 supernatants are screened for toxin-specific
5 antibodies. Hybridomas positive for antibody activity
6 are selected and expanded. Perpetual cell lines can
7 then be maintained according methods well-known in the
8 art.

9 A monoclonal antibody-producing hybridoma was
10 generated in this manner by Strockbine, et al., from
11 the fusion of SP2/0-Ag14 myeloma cells and EALE/c mice
12 immunized with purified, biologically active SLT from
13 *E. coli* H30. This hybridoma was designated 13C4
14 (Strockbine, N.A., Marques, L.R.M., Holmes, R.F. and
15 O'Brien, A.D., 1985, Characterization of Monoclonal
16 Antibodies against Shiga-Like Toxin from *Escherichia*
17 *coli*, *Infection and Immunity*, 50:695-700). This
18 antibody is generally characterized as being of the G1
19 heavy and kappa light chain classes. This hybridoma
20 was deposited at the American Type Culture Collection,
21 12301 Parklawn Drive, Rockville, Maryland 20852, USA
22 and was assigned catalogue number CRL 1794. This
23 hybridoma is hereinafter referenced as ATCC CRL 1794.

24 The monoclonal antibodies against SLT-II are
25 prepared from a toxoid derived from SLT-II toxin.
26 Crude SLT-II toxin is produced from toxin-converting

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1 phage plaque preparations by means of coliphage plaque
2 lawns using techniques known in the art. The crude
3 toxin is then converted to toxoid by treatment with
4 formaldehyde or glutaraldehyde in Na_2HPO_4 and the
5 remaining formaldehyde or glutaraldehyde is removed.

6 Female BALB/c mice are immunized with the toxoid.
7 The mice are then sacrificed and their splenocytes are
8 harvested. The spleen cells are prepared and fused to
9 SP2/O-Ag 14 mouse myeloma cells by methods well-known
10 in the art. The resulting hybridomas are then
11 cultured. The culture supernatants are assayed and
12 those cultures showing positive for antibody activity
13 are selected and expanded. Perpetual cell lines can
14 then be maintained using methods well-known in the art.

15 Two monoclonal antibody-producing hybridomas were
16 generated in this manner by Perera, et al., from the
17 fusion of SP2/O-Ag14 myeloma cells and BALB/c mice
18 immunized with formilized SLT-II toxoid (Perera,
19 L.P., Marques, L.R.M., and O'Brien, A.D., 1988,
20 Isolation and Characterization of Monoclonal Antibodies
21 to Shiga-Like Toxin II of Enterohemorrhagic Escherichia
22 coli and Use of the Monoclonal Antibodies in a Colony
23 Enzyme-Linked Immunosorbent Assay, J. Of Clinical
24 Microbiology 26:2127-2131). One of the hybridomas,
25 designated 11F11, is characterized as being of the IgM
26 class, having a kappa light chain. The other

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1 hybridoma, designated 11E10, is characterized as being
2 of the IgG, subclass with a Kappa light chain. These
3 hybridomas were deposited at the American Type Culture
4 Collection, 12301 Parklawn Drive, Rockville, Maryland
5 20852, USA. Hybridoma 11E10 was assigned catalogue
6 number CRL 1907 and hybridoma 11F11 was assigned
7 catalogue number CRL 1908. Hereinafter these
8 hybridomas will be referenced as ATCC CRL 1907 and ATCC
9 CRL 1908, respectively. Both ATCC CRL 1907 and ATCC
10 CRL 1908 react with the A subunit of SLT-II but not
11 with the B subunit.

12 Bacterial culture samples are spotted onto a
13 nitrocellulose membrane preferably using a dot blot
14 apparatus connected to a vacuum. The membrane is air
15 dried and then incubated in a solution containing a
16 biological detergent and a blocking agent capable of
17 blocking nonspecific binding sites. The solution such
18 as phosphate buffered saline with 0.1% Tween 20 (PBS-T)
19 and 5% non-fat dry milk may be used. After an
20 appropriate period of incubation the membrane is washed
21 to remove excess blocking agent.

22 The membrane is next incubated in a mixture of two
23 monoclonal antibodies (one specific for the SLT-I and
24 the other specific for SLT-II) diluted in PBS-T to
25 allow the antibodies to bind to any SLTs which may be
26 present in the culture samples. The monoclonal

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1 antibody against SLT-I may be any monoclonal antibody
2 which is specific for SLT-I, but generally will be
3 specific for the B subunit of SLT-I. Similarly, the
4 monoclonal antibody against SLT-II may be any
5 monoclonal antibody which are specific for SLT-II, but
6 generally will be specific for the A subunit of SLT-II.
7 In the preferred embodiment, the monoclonal antibodies
8 specific for SLT-I are the antibodies produced by the
9 hybridoma ATCC CRL 1794 and the monoclonal antibodies
10 specific for SLT-II is the antibodies produced by
11 hybridoma ATCC CRL 1907. Dilutions of ATCC CRL 1794,
12 ATCC CRL 1907 and ATCC CRL 1908 in phosphate buffered
13 saline ranging from 1:2 to 1:64 are sufficiently
14 sensitive for the purposes described herein.

15 After appropriate incubation, the monoclonal
16 antibody mixture is removed and the membrane is washed
17 to remove any remaining monoclonal antibodies which
18 have not bound to the samples. The samples are then
19 assayed for the presence of bound antibodies using
20 western blotting techniques and an enhanced
21 chemiluminescent compound.

22 The membrane is incubated in a solution containing
23 a secondary antibody which has been conjugated to a
24 peroxidase enzyme as a label. The secondary antibody
25 is allowed to bind to any anti-SLT antibody present on
26 the membrane. The sample is then washed to remove

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1 unbound secondary antibody. The membrane is immersed
2 in a detection reagent consisting of a chemiluminescent
3 compound, an oxidant and a compound capable of
4 enhancing the luminescing reaction which occurs with
5 peroxidase-catalyzed oxidation of the oxidant in the
6 presence of the chemiluminescent compound. The amount
7 of bound antibody is measured by detecting the
8 luminescence of the sample.

9 Western blotting techniques using a
10 chemiluminescent label are preferred as a rapid, highly
11 sensitive and non-radioactive assay. After exposure to
12 the antibody of the invention, the membrane is
13 incubated in a horseradish peroxidase-conjugated anti-
14 mouse immunoglobulin G antibody and then washed to
15 remove any unbound antibody. The membrane is then
16 immersed in a detection reagent containing an oxidant,
17 a chemiluminescing compound and a phenolic enhancer.

18 The chemiluminescent reaction is a peroxidase-
19 catalyzed reaction of an oxidant and a chemiluminescent
20 compound. In ELISAs, the peroxidase enzyme is
21 conventionally a horseradish peroxidase enzyme which
22 has been conjugated to an anti-mouse immunoglobulin
23 antibody. However, other peroxidases, particularly
24 plant peroxidases, may be substituted. Where each of
25 the heterologous monoclonal antibodies are diluted in
26 the range of 1:2 to 1:64, a horseradish peroxidase-

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1 conjugated goat antimouse immunoglobulin G antibody may
2 be used in a dilution with a physiological buffered
3 saline solution in the range of 1:500 to 1:5000.

4 Chemiluminescent compounds provide a rapid and
5 safe means for conducting immunoassays.
6 Chemiluminescent compounds are converted to an excited
7 state during the oxidation reaction and return to a
8 non-excited state through the emission of light.
9 Chemiluminescent compounds are generally described as
10 being 2,3-dihydro-1,4-phthalazinedione (DPD) compounds
11 capable of emitting light through the previously
12 described oxidation reaction. The most commonly used
13 DPD compounds are luminol (5-amino-2,3-dihydro-1,4-
14 phthalazinedione) and isoluminol (6-amino-2,3-dihydro-
15 1,4-phthalazinedione).

16 Solutions containing chemiluminescent DPD
17 compounds, alone, are not sufficiently sensitive to
18 detect low, but clinically significant, amounts of SLT
19 in samples. The sensitivity of chemiluminescent
20 reaction is therefore enhanced by the addition of a
21 phenol or naphthol having a general formula as
22 described in United States Patent No. 4,598,044 at
23 column 2, line 37 through column 3, line 3 and column
24 4, lines 28-45. The described phenols and naphthols
25 capable of enhancing the sensitivity of the

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1 chemiluminescing reaction are hereinafter referred to
2 as sensitivity enhancers.

3 The oxidant will be selected for its ability react
4 with the predetermined DPD compound, resulting in the
5 emission of light. Commonly used oxidants include
6 hydrogen peroxide and solutions containing perborate
7 ion.

8 After immersion, the membrane is immediately
9 exposed to a photographic film capable of detecting the
10 light emitted from the peroxidase catalyzed reaction of
11 the detection reagent. The exposure time is dependant
12 upon the film used but generally will range from
13 approximately 10 second to 3 minutes. The presence of
14 toxin can then be detected when the film is exposed.

15 Physiologically buffered saline solutions,
16 biological detergents and blocking agents capable of
17 blocking nonspecific binding sites are all well known
18 to the art and practitioners will readily appreciate
19 that a wide range of combinations could be substituted
20 for preferred solution without significantly affecting
21 the sensitivity of the assay. For example, bovine
22 serum albumen (BSA) may be substituted for nonfat dry
23 milk as a blocking agent. Similarly, borate,
24 carbonate, acetate, and Tris
25 [tris(hydromethyl)aminomethane] could be substituted
26 for phosphate as a buffer. Further, any biological

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1 detergent generally having similar properties to Tween-
2 20 may be substituted. The chemiluminescent reaction
3 will generally occur over a range of pH from 6 to 10,
4 but preferably would be within a range of pH 7-9.

5 Enzyme-linked Immunosorbent Assays (ELISAs) are
6 generally-known means of assaying for the presence of
7 antigens in test material. Those familiar with art
8 will readily appreciate that invention described herein
9 may be adapted to other conventional ELISA techniques.
10 For example, the peroxidase enzyme may be conjugated
11 directly to the monoclonal antibodies against SLT-I and
12 SLT-II. In such case, the use of an anti-mouse
13 secondary antibody would be omitted.

14 Specifically, the invention is a diagnostic kit
15 where the two heterologous monoclonal antibodies are
16 used as a probe to carry out the method described
17 above. Such kits would include antibodies against SLT-
18 I and antibodies against SLT-II in aqueous solution,
19 together with an enhanced chemiluminescent detection
20 reagent containing a chemiluminescent DPD and a
21 sensitivity enhancer, which is a phenol or naphthol
22 capable of enhancing the sensitivity of the
23 chemiluminescing reagent.

24 For a convenient, rapid test to determine the
25 presence of any SLT, the two heterologous antibodies
26 may be provided as a mixture in a single aqueous

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1 solution. In other embodiments, however, the kits may
2 include two solutions, one containing only antibodies
3 against SLT-I and the other containing antibodies only
4 against SLT-II.

5 Conventionally, the kits would also include a
6 substrate on which to perform the assay, wash
7 solutions, a secondary antibody capable of binding the
8 previously-described monoclonal antibodies and which is
9 conjugated to a peroxidase enzyme, and film or other
10 means for detection of the light produced by the
11 chemiluminescent reaction, reagents necessary for
12 processing the light detection means, and instructions
13 for the use of the kit. In such conventional kit, the
14 antibody mixtures and all reagents would be provided in
15 standardized dilutions, such that the user would need
16 only to prepare the sample, including serial dilution
17 (if desired), and proceed with the assay according to
18 the directions provided.

19 The chemiluminescent reaction will occur
20 adequately at normal room temperatures. Accordingly,
21 no special apparatus or facility will normally be
22 required for temperature maintenance when using the
23 kit.

24 Although the description and example of the
25 invention provided herein demonstrate a simple kit for
26 detection of SLT in food or clinical samples, those

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1 familiar with art will readily understand that other
2 forms of the kit will allow the user to detect the
3 relative quantity of toxin contained in a sample. By
4 way of example, such a kit may use a means for
5 measuring the amount of light emitted by the
6 chemiluminescent assay of a clinical sample, or the kit
7 may provide for the assay of a sample culture against
8 one or more series of cultures having predetermined
9 quantities of toxin.

10 WORKING EXAMPLE

11 Bacterial cultures are spotted onto BAS-NC™
12 nitrocellulose (Schleicher & Schuell, Inc., Keene New
13 Hampshire) through a 96-well dot blot apparatus
14 (Schleicher & Schuell, Inc.) connected to a vacuum.
15 The nitrocellulose membrane is air-dried and incubated
16 for 1 hour at room temperature in phosphate-buffered
17 saline with 0.1% Tween 20 (PBS-T) (Bio-Rad
18 Laboratories) containing 5% non-fat dry milk (Carnation
19 Co., Los Angeles California). The membrane is washed
20 with PBS-T and then incubated with a mixture of
21 monoclonal antibodies ATCC CRL 1794 [1:5 dilution] and
22 ATCC CRL 1907 [1:5 dilution] in PBS-T for 1 hour. The
23 membrane is washed three times with PBS-T to remove
24 unbound antibody. The membrane is then incubated for 1
25 hour with a 1:500 dilution of horseradish peroxidase-
26 conjugated goat anti-mouse immunoglobulin G antibody

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1 (Bio-Rad Laboratories) in PBS-T. After incubation, the
2 membrane is washed five times in PBS-T, immersed in
3 ECL™ Western blotting detection reagent (Amersham
4 International PLC, Little Chalfont, United Kingdom) for
5 1 minute and then immediately exposed to X-OMAT™ film
6 (Eastman Kodak Company, Rochester, New York). for
7 approximately 3 minutes, after which the film is
8 developed and the presence of toxins detected.

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9 We claim:

- 10 1. A diagnostic kit for the detection of Shiga-
11 like toxins comprising
12 an SLT antibody reagent comprising an antibody
13 specific to Shiga-like toxin, type I, and an
14 antibody specific to Shiga-like toxin, type
15 II, in aqueous solution; and
16 a detection reagent comprising a chemiluminescent
17 2,3-dihydro-1,4-phthalizinedione and a
18 sensitivity enhancer capable of enhancing the
19 sensitivity of the chemiluminescent 2,3-
20 dihydro-1,4-phthalizinedione reaction.
- 21 2. The diagnostic kit of claim 1 wherein the
22 antibody specific to Shiga-like toxin, type I, is ATCC
23 CRL 1794 and the antibody specific to Shiga-like toxin,
24 type II, is ATCC CRL 1907.
- 25 3. The diagnostic kit of claim 1 wherein the
26 antibody specific to Shiga-like toxin, Type I, is ATCC

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1 CRL 1794 and the antibody specific to Shiga-like toxin,
2 Type II, is ATCC CRL 1908.

3 4. The diagnostic kit of claim 1, wherein the
4 chemiluminescent 2,3-dihydro-1,4-phthalizinedione is
5 selected from luminol or isoluminol, and the
6 sensitivity enhancer is selected from 4-iodophenol, 4-
7 phenylphenol or 2-chloro-4-phenylphenol.

8 5. The diagnostic kit of claim 1, having as an
9 additional component a labelling reagent comprising of
10 a horseradish peroxidase labelled antibody directed
11 against the antibodies of the SLT antibody reagent in
12 an aqueous solution.

13 6. The diagnostic kit of claim 1, wherein the
14 detection reagent is further comprised of hydrogen
15 peroxide.

16 7. A diagnostic kit for the detection of Shiga-
17 like toxins comprising

18 an antibody specific to Shiga-like toxin, type I,
19 in aqueous solution;

20 an antibody specific to Shiga-like toxin, type II,
21 in aqueous solution; and

22 a detection reagent comprising a chemiluminescent
23 2,3-dihydro-1,4-phthalizinedione and a sensitivity
24 enhancer capable of enhancing the sensitivity of the
25 chemiluminescent 2,3-dihydro-1,4-phthalizinedione
26 reaction.

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1 8. The diagnostic kit of claim 7, wherein the
2 antibody specific to Shiga-like toxin, type I, is ATCC
3 CRL 1794.

4 9. The diagnostic kit of claim 7, wherein the
5 antibody specific to Shiga-like toxin, type II, is
6 selected from ATCC CRL 1907 and ATCC CRL 1908.

7 10. The diagnostic kit of claim 7, wherein the
8 antibody specific to Shiga-like toxin, type I, is ATCC
9 CRL 1794 and the antibody specific to Shiga-like toxin,
10 type II, is ATCC CRL 1907.

11 11. The diagnostic kit of claim 7, wherein the
12 chemiluminescent 2,3-dihydro-1,4-phthalizinedione is
13 selected from luminol or isoluminol, and the
14 sensitivity enhancer is selected from 4-iodophenol, 4-
15 phenylphenol or 2-chloro-4-phenylphenol.

16 12. The diagnostic kit of claim 7, wherein the
17 detection reagent is further comprised of hydrogen
18 peroxide.

19 13. The diagnostic kit of claim 7, having as an
20 additional component a labelling reagent comprised of a
21 horseradish peroxidase labelled antibody directed
22 against the antibody specific to Shiga-like toxin, type
23 I, and the antibody specific to Shiga-like toxin, type
24 II.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/02664

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/569

US CL : 435/7.37, 7.32, 7.92, 7.95; 530/388.4, 389.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbol)

U.S. : 435/7.37, 7.32, 7.92, 7.95, 968, 975; 530/388.4, 389.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG (file biochem) search terms: shiga, toxin, antibod7, type I, type II, chemilumines7

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Clinical Microbiology, Volume 26, No. 10, issued October 1988, L.P. Perera et al, "Isolation and Characterization of Monoclonal Antibodies to Shiga-Like Toxin II of Enterohemorrhagic Escherichia coli and Use of the Monoclonal Antibodies in a Colony Enzyme-Linked Immunosorbent Assay," pages 2127-2131, especially the first full paragraph of page 2128, the paragraph bridging pages 2129 and 2130, the first full paragraph in column 1 of page 2130, and Table 2.	1-13
Y	US, A, 4,598,044 (KRICKA ET AL) 01 JULY 1986, column 1, lines 5-7; column 2, line 37-column 3, line 3; column 4, lines 11-26; column 4, lines 34-49; column 5, lines 5-10; and column 8, lines 40-68.	1-13

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* Z* document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 MAY 1996

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24 MAY 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/02664

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Seventh Edition, issued 1992, R. Hay et al, editors, pages 340, 341 and 346.	1-13
Y	Amersham Life Sciences Products Catalog, Amersham Corporation, Arlington Heights, Illinois, issued 1992, pages 68-69.	1-13
Y,P	Infection and Immunity, Volume 50, No. 3, issued December 1995, N.A. Strockbine et al, "Characterization of Monoclonal Antibodies against Shiga-Like Toxin from Escherichia coli," pages 695-700, especially the first and second paragraphs of page 695.	1-13